Chapter 4

In Vitro and In Vivo Methodologies for Studying the Sigma 54-Dependent Transcription

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Abstract

Here we describe approaches and methods to assaying in vitro the major variant bacterial sigma factor, Sigma 54 (σ^{54}), in a purified system. We include the complete transcription system, binding interactions between σ54 and its activators, as well as the self-assembly and the critical ATPase activity of the cognate activators which serve to remodel the closed promoter complexes. We also present in vivo methodologies that are used to study the impact of physiological processes, metabolic states, global signalling networks, and cellular architecture on the control of σ^{54}-dependent gene expression.

Key words Transcription activation, RNA polymerase, σ^{54}, Open and closed promoter complexes, AAA+ proteins, Bacterial enhancer binding proteins, ATPase

1 Introduction

The σ^{54} transcription system is distinctive amongst bacterial gene regulation systems in that the RNA polymerase (RNAP) closed promoter complex (RP_C) fully forms, and then remains stably associated with promoter DNA until remodelled by its cognate AAA+ activator proteins in an ATP hydrolyzing reaction (Fig. 1). The ATP-dependent remodelling of σ^{54} within RP_C yields the open promoter complex RP_O, from which transcripts can be made [1] (Fig. 1). Since the activators work from remote upstream enhancer-like sites, they are called bacterial enhancer binding proteins (bEBPs). This property together with the involvement of ATP in the DNA melting process draws obvious comparison with the more complex eukaryotic transcription control of Pol II.

Most in vitro studies of σ^{54}-dependent transcription have been conducted with components from *Escherichia coli*, *Salmonella*, *Aquifex aeolicus*, *Rhizobium* sp., or *Pseudomonas* sp. bacteria (not

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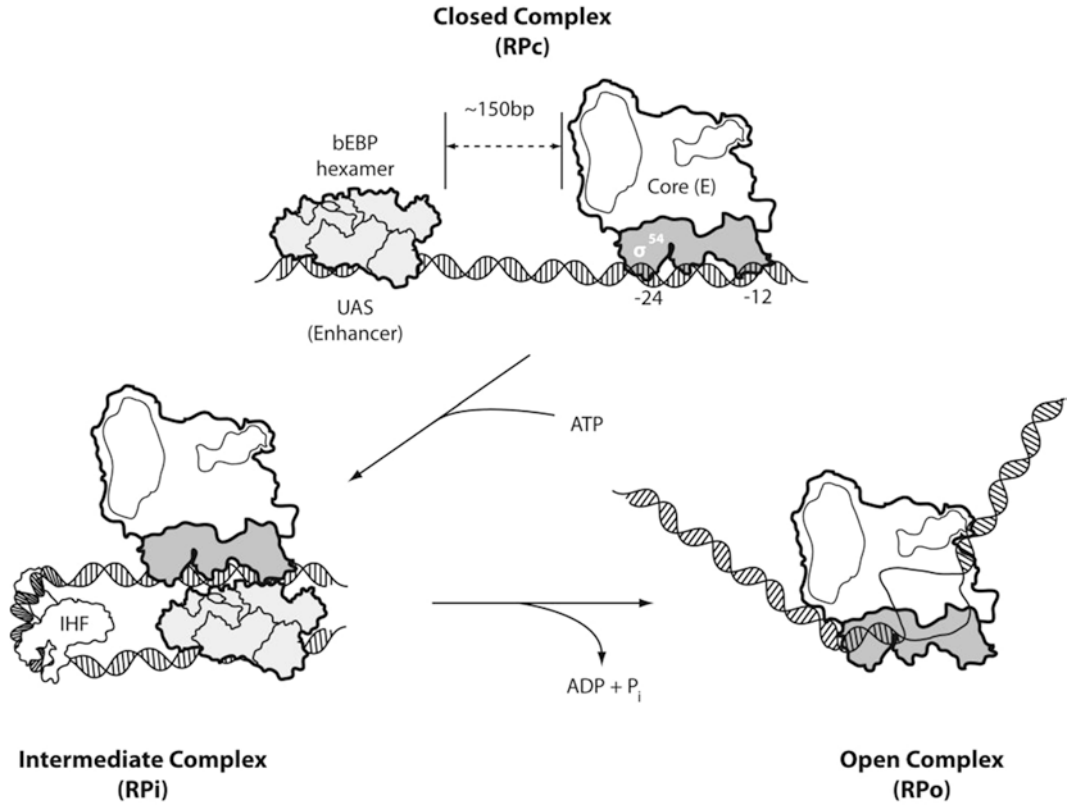


Fig. 1 The gene transcription activation by σ^{54}-holoenzyme ($E\sigma^{54}$). The enzyme RNA polymerase containing σ^{54} factor binds to −12 and −24 promoter sites forming a closed complex (RPc) due to a transient fork junction located at the −12 site. A bEBP complex bound to an Upstream Activation Sequence (UAS) ~150 bp upstream of the start site contacts σ^{54} via a DNA looping event facilitated by for example the Integration Host Factor (IHF), thereby forming the intermediate complex (RPi). Energy derived from the ATPase activity of the bEBP is used to remodel the promoter complex into an open complex (RPo)

an exhaustive list), and often heterologous systems have been used to evaluate activities of individual components. Below we describe one *E. coli* system employing the bEBP Phage Shock Protein F (PspF) [2], and one employing the *Pseudomonas syringae* co-dependent HrpRS bEBPs [3]. For PspF, its AAA+ domain (here we use amino acids 1–275) is sufficient for stimulating formation of RP_Os, whereas the full-length HrpR and HrpS need to be co-expressed to form an active HrpRS assembly. When studying bEBPs with conventional receiver domains of the 2CR family, deleting these domains, phosphorylating them using either e.g., carbomyl phosphate or their cognate histidine protein kinase, or introducing a phosphor-mimic amino acid substitution would be necessary for obtaining active self-assemblies.

Although we describe approaches with purified components that can yield important inroads into using structural biology to help establish mechanism [4–7], much can also be gained using in vivo methods such as promoter fusions, two hybrid approaches [3] and single molecule imaging [8] for establishing determinants of component interactions and assembly, and localizations and dynamics of protein or protein–DNA complexes. In vivo methods not only allow for recapitulating biochemical data in the context of the living cell but may also reveal new regulatory features which cannot be obtained through in vitro experimentation. The methods presented here are transferable to many AAA+ bEBP proteins.

2 Materials

2.1 Equipment

1. Protein purification system.
2. High-speed centrifuge, such as Beckman Avanti with JA-14 and JA-25.50 rotors (or comparable).
3. Sonicator.
4. Tabletop centrifuge.
5. SDS polyacrylamide gel electrophoresis (PAGE) apparatus.
6. Sequencing gel apparatus.
7. Power supplies.
8. Thin-layer chromatography (TLC) chamber.
9. A 4 °C cooled flat surface.
10. A short wavelength UV light source (e.g., 254 nm, e.g., UVG-54, UVP Inc., CA).
11. Phosphor-Image reader, screens, and suitable quantification software; we use Fuji-Bas 1500 and Aida, respectively.
12. Facilities and training for safe working with ^{32}P isotopes.

2.2 E. coli Cell Growth, Lysis, and Purification of Core RNAP and Klebsiella pneumoniae σ^{54}

1. LB medium, Luria–Bertani broth per 1 l dH_2O: 10 g peptone (or tryptone), 5 g yeast extract, 5 g NaCl.
2. 1 M isopropyl-beta-D-thiogalactopyranoside (IPTG) in dH_2O.
3. Lysis Buffer: 20 mM Tris–HCl pH 8.0, 500 mM NaCl, 5 % glycerol.
4. Buffer A-1: 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 5 % glycerol.
5. Buffer B-1: 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 1 M imidazole, 5 % glycerol.
6. Dialysis Buffer-1: 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5 % glycerol.
7. Buffer A-2: 50 mM Tris–HCl pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 5 % glycerol.

8. Buffer B-2: 50 mM Tris–HCl pH 8.0, 1 M NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, 5 % glycerol.
9. Dialysis Buffer-2: 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 50 % glycerol.

2.3 Purification of *E. coli* $PspF_{1–275}$

1. Buffer A: 25 mM NaH_2PO_4 pH 7.0, 500 mM NaCl, 5 % glycerol.
2. Buffer B: 25 mM NaH_2PO_4 pH 7.0, 500 mM NaCl, 1 M imidazole, 5 % glycerol.
3. Dialysis Buffer: 20 mM Tris–HCl pH 8.0, 200 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 5 % glycerol.

2.4 Purification of *Pseudomonas sp.* HrpR and HrpS

1. Buffer A: 50 mM sodium phosphate buffer pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 5 % glycerol.
2. Buffer B: 50 mM sodium phosphate buffer pH 7.0, 50 mM NaCl, 0.1 mM EDTA, 1 M imidazole, 5 % glycerol.
3. Dialysis Buffer: 20 mM Tris pH 8.0, 50 mM NaCl, 0.1 mM EDTA, and 5 % glycerol.

2.5 In Vitro Full-Length and Abortive Transcription Assays

1. 1× TM Buffer: 10 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$.
2. 1× STA Buffer: 2.5 mM Tris-Acetate pH 8.0, 8 mM Mg-Acetate, 10 mM KCl, 1 mM DTT, 3.5 % PEG 8000.
3. 1× TTH Buffer: 10 mM Tris–HCl, pH 7.5, 70 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM EDTA, 12.5 % glycerol, and 0.1 % w/v Triton X-100.
4. Nucleotide mixture: 0.2 mM ATP, 0.1 mM GTP, 0.1 mM CTP, 0.05 mM UTP.
5. 3× Formamide Stop Dye: 3 mg xylene cyanol, 3 mg bromophenol blue, 0.8 ml 250 mM EDTA, 10 ml deionized formamide for a final volume of 10 ml.
6. 4 % sequencing gel: 8 ml commercial UreaGel Concentrate, 37 ml commercial SequaGel Diluent, 5 ml 10× TBE Buffer, 500 µl 10 % APS, and 40 µl TEMED.
7. 1× TBE Buffer: 0.089 M Tris base, 0.089 M boric acid pH 8.3, 2 mM Na_2 EDTA.
8. 20 % sequencing gel: 20 ml commercial UreaGel Concentrate, 2.5 ml commercial SequaGel Diluent, 2.5 ml 10× TBE, 200 µl 10 % APS, 20 µl TEMED.

2.6 Trapping Assay with Nucleotide Analogues

1. 5× Native Loading Dye: 10 % glycerol, 0.5 mg bromophenol blue.
2. 4.5 % native gel: 0.75 ml acrylamide 37.5:1 (30 % acrylamide–0.8 % bis-acrylamide), 0.5 ml 10× TG, 3.75 ml H_2O, 50 µl 10 % APS.
3. 1× TG Buffer: 0.025 M Tris base, 0.192 M glycine pH 8.4.

2.7 ATPase Assay Using TLC

1. 5× ATPase Buffer (e.g., for PspF and HrpRS): 20 mM Tris–HCl pH 8.0, 50 mM NaCl, 15 mM $MgCl_2$, 0.1 mM EDTA, 10 μM DTT. (e.g., for NtrC bEBP as well as for PspF, one can use the buffer: 35 mM Tris-acetate, pH 8.0, 70 mM potassium-acetate, 15 mM magnesium acetate, 19 mM ammonium acetate, 0.7 mM DTT.)
2. ATP mixture: Unlabelled ATP and [α-^{32}P] radiolabelled ATP with sufficiently high specific activity (e.g., 0.6 μCi/μl).
3. TLC running buffer: 0.4 M K_2HPO_4, 0.7 M boric acid.

2.8 UV Cross-Linking of Bound Nucleotides to Estimate ATP Binding

1. UV cross-linking buffer: 35 mM Tris-acetate, pH 8.0, 70 mM potassium-acetate, 15 mM magnesium acetate, 19 mM ammonium acetate, 0.7 mM DTT.
2. [α-^{32}P] and/or [γ-^{32}P] radiolabelled ATP must be of high specific activity (e.g., 0.6 μCi/μl and 3,000 Ci/mmol).

2.9 UV Cross-Linking Using the APAB Cross-Linker

1. Cross-linking dye: 0.25 M Tris–HCl pH 6.8, 25 % glycerol, 5 % SDS, 5 % β-mercaptoethanol, 0.5 g bromophenol blue, 5 M deionized urea.
2. 7.5 % SDS gel: 2.5 ml acrylamide 37.5:1 (30 % acrylamide–0.8 % bis-acrylamide), 2.5 ml Solution II, 5 ml H_2O for the resolving gel.

2.10 Determining Proteins Oligomeric State by Gel Filtration

1. Gel filtration buffer: 20 mM Tris–HCl (pH 8), 50 mM NaCl and 15 mM $MgCl_2$.

2.11 Transcriptional Reporter

1. Z buffer: 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol.

2.12 In Vivo Footprinting

1. TMD: 500 mM Tris–HCl pH 7.2, 100 mM $MgSO_4$, 2 mM DTT.
2. Primer extension mix: 5 mM $MgCl_2$, 5 mM Tris–HCl (pH 8.0), 1 mM of each dNTP, and 0.5 unit of Klenow fragment of DNA polymerase I.

2.13 Analysis of Protein Dynamics

1. Minimal growth medium: 50 mM MOPS, 2 mM $MgSO_4$, 0.7 mM Na_2SO_4, 1.2 mM NH_4NO_3, 0.5 mM KH_2PO_4, 10 mM NH_4Cl, 0.4 % w/v glucose (filter sterilized), 1× trace elements.

3 Methods

3.1 Preparation of E. coli Core RNAP

It is convenient to use the pVS10 plasmid (*rpoA-proB-rpoC* [His-tag]-*rpoZ*) [9] to obtain overexpressed core RNAP; *see* also Chapter 2 in this volume.

1. Freshly transformed *E. coli* NovaBlue cells harboring the pVS10 plasmid are used to set up a 25 ml overnight culture in LB medium at 37 °C supplemented with ampicillin (100 μg/ml).
2. For overexpression, 20 ml of the overnight culture are used to inoculate 1 L of LB medium at 37 °C with ampicillin (100 μg/ml) with vigorous shaking.
3. At $OD_{600nm} = 0.4$, the culture is cold-shocked on ice for 20 min and then induced with 1 mM IPTG at 37 °C for 3 h. Cells are harvested by centrifugation (5,000 rpm or 4,420 × *g*, 15 min at 4 °C in a Beckman JA-14 rotor).
4. The cell pellet is resuspended in cold Lysis Buffer (20 ml Lysis Buffer per 1 l cell pellet) and disrupted by two rounds of sonication on ice (40 % energy, 2 s on/off pulse for 10 min in a SONICS Ultra Cell).
5. The lysate is centrifuged (14,000 rpm or 20,617 × *g*, 30 min at 4 °C in a Beckman JA-25.50 rotor) and half a tablet of the protease inhibitor cocktail (Roche) is added to the supernatant.
6. The supernatant is immediately loaded onto two pre-equilibrated HiTrap 5 ml NiNTA chelating columns on an AKTA or equivalent chromatography system at a flow rate of 0.5 ml/min. Prior to sample loading, the two chelating columns are washed with three volumes of filtered water and equilibrated with two volumes of Buffer A-1.
7. The protein-bound columns are washed with three volumes of 0 % Buffer B-1 at a 2 ml/min rate, followed by another three volumes of 3 % Buffer B-1 at 2 ml/min.
8. Proteins are eluted in 1 ml fraction size with a linear Buffer B-1 gradient up to 100 % in 40 min at 1 ml/min.
9. After analyzing on SDS-PAGE, fractions containing core RNAP are exchanged overnight at 4 °C with Dialysis Buffer-1 in a dialysis tube (MWCO 30 kDa) and stored at −80 °C.
10. Core RNAP is then subjected to a second round of purification. 10 ml of $MgCl_2$ are added to the dialyzed protein sample before being loaded onto two HiTrap 5 ml Heparin columns pre-equilibrated with Buffer A-2 on an AKTA or equivalent chromatography system (*see* **Note 1**).
11. The protein-bound columns are washed with 0 % Buffer B-2 for three volumes and eluted in 1 ml fraction size at a linear Buffer B-2 gradient to 100 % in 60 min at 1 ml/min.
12. The SDS-PAGE analyzed fractions are dialyzed overnight at 4 °C in Dialysis Buffer-2 and stored at −80 °C.

3.2 Preparation of Klebsiella pneumoniae σ^{54}

For historical reasons, we used the σ^{54} protein from *K. pneumoniae* which is essentially interchangeable in functionality with the *E. coli* σ^{54} [10]. The *K. pneumonia* σ^{54} can be expressed in high yields using the T7 promoter-controlled pET28b+ vector in *E. coli* BL21 (DE3) cells.

1. Freshly transformed cells are used to inoculate 1 l of LB medium supplemented with kanamycin (50 μg/ml) at 37 °C with vigorous shaking.
2. At $OD_{600nm} = 0.4$, the cell culture is cold-shocked on ice for 20 min before induced with 1 mM IPTG at 37 °C for 3 h.
3. Cells are harvested by centrifugation (5,000 rpm or 4,420 × *g*, at 4 °C in a Beckman JA-14 rotor) and resuspended in cold Lysis Buffer (20 ml Lysis Buffer per 1 l of cell pellet).
4. Cells are disrupted by two rounds of sonication on ice (40 % energy, 2 s on/off pulse for 10 min).
5. Cell debris is removed by centrifugation (14,000 rpm or 20,617 × *g*, 30 min at 4 °C in a Beckman JA-25.50 rotor) and the supernatant is supplemented with half a tablet of protease inhibitor cocktail.
6. The soluble protein is loaded onto two HiTrap 5 ml NiNTA chelating columns pre-equilibrated with Buffer A-1.
7. After three volumes of 0 % Buffer B-1 wash followed by three volumes of 3 % Buffer B-1 wash, proteins are eluted in 1 ml fraction size at 1 ml/min under a linear Buffer B-1 gradient to 100 % in 40 min.
8. Fractions containing proteins are dialyzed in Dialysis Buffer-1 in a dialysis tube (MWCO 12–14 kDa) and stored at −80 °C.
9. The NiNTA-purified *K. pneumoniae* σ^{54} is cleaved free of His-tag using a Thrombin Cleavage Capture Kit and subject to Heparin purification.
10. The dialyzed samples are loaded onto two HiTrap 5 ml Heparin columns pre-equilibrated with Buffer A-2 on an AKTA or equivalent chromatography system.
11. The protein-bound columns are washed with 0 % Buffer B-2 for three volumes and eluted in 1 ml fraction size at a linear Buffer B-2 gradient to 100 % in 60 min at 1 ml/min.
12. Proteins are dialyzed in Dialysis Buffer-2 and stored at −80 °C.

3.3 Preparation of E. coli $PspF_{1–275}$

1. The *E. coli* bEBP $PspF_{1–275}$ [11] is cloned into pET28b+ vector plasmid and overexpressed in an identical procedure to that used for σ^{54} (*see* above).
2. The $PspF_{1–275}$ protein is subject only to NiNTA affinity chromatography using Buffer A and 7 % Buffer B for washing and

a 100 % Buffer B linear gradient (in 40 min at a 1 ml/min flow rate) for elution.

3. The fractions containing $PspF_{1-275}$ are cleaved free of His-tag, dialyzed in Dialysis Buffer and stored at −80 °C.

3.4 Preparation of Pseudomonas sp. HrpR and HrpS

HrpRS proteins are expressed in high yields using the pQE70 highly expression vector in *E. coli* B834 cells. The *hrpRS* genes are cloned as operon under the strong T5 (IPTG inducible) promoter. HrpS has a 6×His tag at the C-terminus and HrpR is co-expressed and co-purified with $HrpS_{6\times His}$. The high transcription rate initiated at the T5 promoter is efficiently repressed by the presence of the *lac* repressor protein expressed from pREP4 vector (originally kanamycin resistance, our vector pREP4-Spc has inserted spectinomycin (Spc) cassette and therefore is Spc resistant).

1. Freshly transformed cells are used to inoculate 0.5 l of LB medium supplemented with Amp (100 μg/ml) and spectinomycin (50 μg/ml) at 25 °C with vigorous shaking. a. At $OD_{600nm} = 0.4–0.6$, the cell culture is cold-shocked on ice for 20 min before induced with 0.3 mM IPTG at 16 °C for 16 h.
2. Cells are harvested by centrifugation (5,000 rpm or 4,420 × *g*, at 4 °C in a Beckman JA-14 rotor) and resuspended in cold Buffer A supplemented with protease inhibitor cocktail (20 ml Buffer A per 0.5 l cell pellet).
3. The resuspended cell pellets are disrupted by two rounds of sonication on ice (40 % energy, 2 s on/off pulse for 10 min).
4. Cell debris is removed by centrifugation (14,000 rpm or 20,617 × *g*, 30 min at 4 °C in a Beckman JA-25.50 rotor) and the soluble protein is loaded onto HiTrap 5 ml NiNTA chelating columns pre-equilibrated with Buffer A.
5. After 6–10 volumes of 0 % Buffer B wash, followed by 6–10 volumes of 7 % Buffer B wash, proteins are eluted in 1 ml fraction size at 1 ml/min under a linear Buffer B gradient to 100 % in 40 min.
6. Fractions containing proteins are dialyzed in Dialysis Buffer in a dialysis tube (MWCO 12–14 kDa) and stored at −80 °C.

3.5 Preparation of Promoter Templates for In Vitro Transcription

These are either plasmids based on pLA4 [12] or linear synthetic DNA duplexes used to allow the incorporation of photoreactive probes, unpaired regions or changes to start site sequences. Our usual test promoter is the *Sinorhizobium meliloti nifH* promoter. The sequence for the *nifH* non-template strand from −60 to +28 is: 5′-GAAAGAAAGCCGAGTAGTTTTATTTCAGACGGCTGG CACGACTTTTGCACGATCAGCCCTGGGCGCGCATGC TGTTGCGCATTCATGT-3′ (the consensus −24 "GG" and −12 "GC" elements are underlined).

A minimal scaffold contains an 8 bp RNA/DNA hybrid, an 18 bp downstream duplex and a gap immediately after the $i+1$ site to accommodate the kink. Such organization mimics the DNA/RNA in the crystal structure of the elongation complex [13] and is particularly suitable for studying σ-independent RNA polymerization. The oligonucleotide sequences for the minimal scaffold are:

RNA, 8 nt: 5′-GUAGCGGA-3′.

Template strand DNA, 28 nt: 5′-GGTCCTGTCTGAAATTG TTATCCGCTAC-3′.

The non-template strand DNA, 18 nt: 5′-ACAATTTCAGA CAGGACC-3′.

1. DNA end-labelling reactions are carried out in 20 μl volumes, containing: 1 μM single-stranded linear DNA, 1 μl of [γ-^{32}P] ATP, and 1 unit of T4 nucleotide kinase at 37 °C for 30 min. Then the T4 nucleotide kinase is heat-deactivated at 65 °C for 10 min.
2. To form stable DNA duplexes, ^{32}P-labelled (or unlabelled) linear DNA is mixed with its unlabelled complementary strand in 1× TM Buffer (*see* Subheading 2.5), heated at 95 °C for 5 min and cooled gradually at room temperature. The DNA/RNA minimal scaffold is formed by the same procedure. These linear probes are used in transcription and trapping assays described below.

3.6 In Vitro Full-Length Transcription Assay

This assay measures a full-length RNA synthesis from a target promoter using the full complement of rNTPs and α-^{32}P labelled UTP.

1. The reactions are performed in 10 μl volumes, containing: 1× STA Buffer, 4 μM $PspF_{1–275}$, 100 nM RNAP holoenzyme (1:4 ratio of E:σ^{54}), 20 units of RNase Inhibitor (e.g., from Promega), 5 % glycerol, 4 mM dATP, and 20 nM DNA at 37 °C. [For HrpR-HrpS dependent transcription (0.8 μM HrpRS for the *hrpL* promoter or 2 μM HrpRS for the *nifH* promoter with addition of 20 nM IHF purified as described in ref. 14 reactions are performed as above but in 1× TTH Buffer and at room temperature (RT); HrpRS are temperature sensitive.]
2. Transcription with $PspF_{1–275}$ is typically activated for 10 min at 37 °C [or for HrpR-HrpS at RT for 20 min (*hrpL*) or 1 h (*nifH*)]. To allow elongation to take place and to prevent further activation, a nucleotide mixture, 0.07 μCi/μl [α-^{32}P]UTP (3,000 Ci/mmol) and 0.1 mg/ml heparin are added to the reaction for 10 min at 37 °C (or for HrpR-HrpS for 15–20 min at RT).
3. Reactions are quenched by 4 μl of 3× formamide stop dye and heated at 95 °C for 5 min.

4. 7 μl of each sample is loaded on a 4 % 35 × 43 cm sequencing gel.
5. The gel is run at 50 W for 1.5–2 h in 1× TBE Buffer in a Bio-Rad Sequi-Gen system until the dye reaches the bottom of the gel, transferred to filter paper, and dried.
6. The dried gel is exposed to an IP imaging plate overnight and visualized; we use a Fuji Base-1500 PhosphorImager.

3.7 In Vitro Abortive Assay

This assay measures the synthesis of short primed RNA (spRNA) created with a −1 + 1 (UpG) or +1 + 2 (GpG) dinucleotide RNA primer and an appropriate (e.g., +2) α-^{32}P labelled rNTP to yield a 3–4-mer for the *nifH* promoter (UpGpGpG or GpGpG).

1. The reactions are typically carried out in a similar manner to the full-length transcription assay. In a 10 μl volume, 1× STA Buffer, 4 μM $PspF_{1–275}$, 100 nM holoenzyme (1:4 ratio of E:σ^{54}), 20 units of RNase Inhibitor, 5 % glycerol, 4 mM dATP, and 20 nM DNA are incubated at 37 °C.
2. After a 10 min activation, elongation mixture containing 0.5 mM initiating dinucleotide primers, 0.2 μCi/μl [α-^{32}P] GTP (3,000 Ci/mmol), and 0.2 mg/ml heparin is added.
3. After incubation for 10 min at 37 °C, the elongation is quenched by 4 μl of 3× formamide stop dye and heated at 95 °C for 5 min.
4. 7 μl of each sample is loaded on a 20 % sequencing gel.
5. The denaturing gel is run under 300 V for 35 min, directly exposed to an IP plate for 10 min. Transcripts are visualized and quantified.

3.8 In Vitro spRNA Assay Using HrpR-HrpS bEBPs

1. This assay is performed with a −1 + 1 UpA dinucleotide RNA primer and an α-^{32}P labelled rNTP to yield a 3 mer for the *hrpL* promoter UpApC. In a 10 μl volume, 1× TTH Buffer, 0.8 μM HrpR-HrpS, 100 nM holoenzyme (1:4 ratio of E:σ^{54}), 20 units of RNase Inhibitor, 4 mM dATP, and 20 nM DNA are incubated at RT.
2. After a 40 min activation, elongation mixture containing 0.5 mM initiating dinucleotide primers, 0.2 μCi/μl [α-^{32}P] GTP (3,000 Ci/mmol), and 0.2 mg/ml heparin is added.
3. After incubation for 15 min at RT, the elongation is quenched by 4 μl of 3× formamide stop dye and heated at 95 °C for 5 min.
4. 7 μl of each sample is loaded on a 20 % sequencing gel. The denaturing gel is run under 300 V for 35 min, directly exposed to an IP plate for 10 min. Transcripts are visualized and quantified.

3.9 Trapping Assay with Nucleotide Analogues

To visualize stable interactions between the holoenzyme (±DNA) and $PspF_{1–275}$ on a native gel, various nucleotide analogues mimicking different hydrolyzing states of ATP are used (e.g., AMP-AlF_x for the ground state and ADP-AlF_x for the transition state).

1. In 10 μl volumes, 2.35 μM σ^{54}, 0.3 μM core RNAP, and (±) 50 nM radiolabelled DNA were pre-incubated with 1× STA Buffer (*see* Subheading 2.5), 5 mM NaF and nucleotides (4 mM ADP or AMP) at 37 °C for 5 min to allow RP_C formation.
2. 10 μl of $PspF_{1–275}$ is added to the reaction to allow contact between bEBP and Eσ^{54}-DNA for 5 min at 37 °C.
3. Subsequently, 0.4 mM $AlCl_3$ is added to allow the in situ formation of nucleotide analogues for 20 min at 37 °C.
4. Samples are mixed with 2.5 μl of 5× Native Loading Dye, loaded on a 4.5 % native gel and run at 150 V for 30 min in 1× TG Buffer.
5. Non-radiolabelled protein complexes are visualized using Sypro Ruby Stain (e.g., from Invitrogen) according to the manufacturer's protocol.
6. When radiolabelled DNA is used, native gels are dried, developed for 1 h and analyzed.

3.10 Supershift σ^{54}-DNA Isomerization Assay

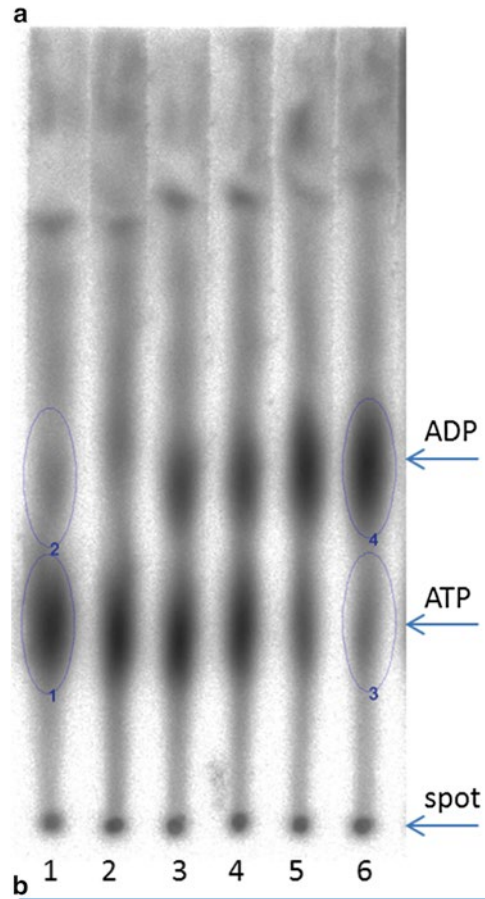
In the presence of a hydrolysable nucleotide (ATP or dATP), $PspF_{1–275}$ is able to isomerize a binary σ^{54}-DNA complex (the early melted linear DNA probe harboring a –12–11 mismatch on the non-template strand to mimic the fork junction DNA) to a conformation with an extended DNase I protection [15]. This isomerization process is thought to proceed the full DNA melting seen in RPo and can generate a super-shifted band on a native gel.

1. In a 10 μl reaction volume, 10 μM $PspF_{1–275}$, 4 mM dATP, 2.35 μM σ^{54}, and 50 nM radiolabelled DNA are incubated in 1× STA Buffer (*see* Subheading 2.5) at 37 °C for 15 min.
2. Samples are mixed with 2.5 μl of 5× Native Loading Dye before loaded on a 4.5 % native gel (*see* Subheading 2.6).
3. Gels are dried, developed for 1 h, and scanned by PhosphorImager.

3.11 ATPase Assay Using TLC

The ATPase catalytic function of bEBPs resides in the AAA+ domain although GTP can also be hydrolyzed and supports transcriptional activation, at least in vitro [16]. bEBPs seem to exclusively hydrolyze ATP to ADP and Pi, since the apparent ADP hydrolysis of the AAA+ domain of NtrC1 from the thermophilic bacterium is attributable to a contaminating adenylate kinase [17]. Triphosphate nucleotide hydrolysis and binding can be measured using ^{32}P labelled nucleotides, described here (and *see* Subheading 3.13), respectively.

Following incubation of bEBPs with a mixture of ATP and ^{32}P labelled ATP, hydrolysis can be directly measured by separating P_i, AMP, ADP, ATP using thin-layer chromatography (TLC) followed by the quantification of resulting ^{32}P labelled species by Phosphor-Imaging (Fig. 2). [α-^{32}P]ATP and [γ-^{32}P]ATP have both been used as nucleotide tracers to determine kinetic parameters of bEBPs,



b

No	Area [mm^2]	Intensity [PSL]
1	267.4	24220.7
2	267.4	3380.8
3	267.4	3397
4	267.4	25552.8

Fig. 2 Thin-layer chromatograph and quantification following [α-^{32}P]ATP hydrolysis by $PspF_{1–275}$. (**a**) Chromatograph showing migration pattern of ATP and ADP after ATP hydrolysis by $PspF_{1–275}$ for 0, 2, 5, 15, and 60 min (*lanes 2–6*) at 37 °C, and in the absence of $PspF_{1–275}$ (*lane 1*). The *numbered blue ovals* indicate evaluation areas used for quantification of phospho-stimulated luminescence (PSL). (**b**) Table of intensity levels (in PSL) obtained for evaluation areas shown in (**a**)

although given good resolution between ATP and ADP during TLC, the use of [α-^{32}P]ATP as substrate may offer the advantage of more accurate quantification of [α-^{32}P]ATP and [α-^{32}P]ADP during Phosphor-Imaging compared with [γ-^{32}P]ATP and $^{32}P_i$ (Fig. 2).

The procedure described here was adapted from Babst et al. [18] to account for the specific buffer requirements for transcription activity of PspF and its relatively low ATPase activity [19]. Special considerations have to be taken into account when determining kinetic parameters for PspF and presumably other bEBPs, whose ATP hydrolysis rates depend on nucleotide binding stimulated assembly into ATPase competent higher order oligomers and nucleotide dependent allosteric subunit hydrolysis effects ([20] and *see* **Note 2**).

Typically, an ATPase assay is performed in the lower micromolar range of protein (monomer) in ATPase buffer and the reaction started by the addition of the mixture ATP in the millimolar range (the cellular concentration of ATP is estimated to be between 1 and 3 mM [21].

3.11.1 Hydrolysis Reaction

This example procedure allows for determining ATP hydrolysis rates during a time course experiment (maximally five time points) at a protein concentration of 20 μM with a starting ATP concentration of 1 mM.

Preparation of ATP mix (for 50 samples): mix 50 μl of 10 mM ATP, 47 μl of H_2O, and 3 μl of [α-^{32}P]ATP (10 μCi/μl, 3,000 Ci/mmol; *see* **Note 3**).

1. Add 5 μl of 40 μM protein to microtube, add 2 μl of 5× ATPase buffer, add 1 μl of H_2O, start reaction by adding 2 μl of ATP mix, record time (*see* **Note 4**).
2. Remove 2 μl of the reaction at desired time points into 10 μl 2 M formic acid, stopping the reaction.

3.11.2 Thin-Layer Chromatography

TLC plate preparation: polyethyleneimine impregnated cellulose absorbant on polyester sheets (20 cm × 20 cm) separate ATP, ADP, AMP, and P_i, following the below procedure. On the absorbant surface of the TLC sheet, with a soft pencil as to not damage the absorbant, draw equal width vertical lane demarcation lines and one horizontal line 3 cm above the edge of the sheet to guide spotting of samples (*see* **Note 5**).

1. Spot 2 μl of the above stopped reactions on the vertical line, side by side for each sample. Samples are loaded approximately 3 cm above the bottom edge of the TLC plate (Polyethyleneimine, PEI, cellulose plate) (*see* **Note 6**).
2. Let air-dry for about 20 min (*see* **Note 7**).
3. Fill a TLC tank or any other suitable transparent glass container (e.g., beaker) with TLC running buffer (eluent), so that eluent depth does not exceed 2 cm.

4. Bend the sheet with the absorbant inwards so that the vertical edges nearly touch and use adhesive strips on the polyester sheet to fix this semi-roll.
5. Stand the semi-roll in the eluent with the spotted samples on the bottom, making sure the spotted samples are about 1 cm above the eluent.
6. Close the container to allow eluent vapors to saturate in the container (otherwise separation is poor).
7. Allow the eluent to migrate upwards by capillary flow until it reaches the top of the sheet (visibly wet, migration takes 2–3 h). The eluent should drive the complete separation of radiolabelled ADP from radiolabelled ATP (*see* **Note 8**).
8. Dry sheet (TLC plate) in air at RT (*see* also **Note 7**).
9. Cover TLC sheet in cling film (to protect the imaging plate).
10. Expose TLC sheet to imaging plate for 1–14 h (*see* **Note 9**).

3.11.3 Experimental Design and Data Analysis

The ATPase assay using TLC in conjunction with ^{32}P labelled NTPs is highly sensitive and accurate. Highly purified proteins should be used since any contaminating ATPase activities will generate misleading results, especially for bEBPs with a low k_{cat} (<1/s). A suitable control is to show that the bEBP mutated in its Walker B motif and purified as wild type has a largely reduced ATPase activity [19].

Figure 2a shows that there is considerable radioactive background along the lanes, presumably because of impurities. This, however, is of no major concern when including a negative control lacking protein (Fig. 2a, lane 1). One analytical way to minimize potential errors that may arise from background and/or inaccurate pipetting is to internally normalize signals by calculating the ADP formation from the imaging signal ratio between ADP and the sum of ADP and ATP ($PSL_{ADP}/(PSL_{ATP}+PSL_{ADP})$), multiplied by the initial known ATP concentration (*see* **Note 10**).

3.12 ATPase Assay Using NADH-Coupled System

The steady-state ATP hydrolysis for $PspF_{1–275}$ can be measured by adopting an NADH-coupled regeneration system [22]. The rate of NADH absorbance decrease at 340 nm is proportional to the rate of steady-state ATP hydrolysis. Automation of this assay allows investigation of multiple protein/ATP concentrations on the same 96-well plate.

1. The ATPase activity of $PspF_{1–275}$ is measured at 37 °C in 100 µl volumes of ATPase reaction: 25 mM Tris–HCl pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM NADH (freshly made), 10 mM phosphoenolpyruvate, 10 U/ml pyruvate kinase, 20 U/ml lactate dehydrogenase, ATP (from 0 to 50 mM), and $PspF_{1–275}$ (from 0 to 20 µM).

2. Samples are transferred onto a 96-well plate (with a lid to prevent volume loss) and analyzed. We use a FLUOstar Omega spectrometer (sourced BMG Labtech) at 37 °C (98 for No. of kinetic cycles, 80 s for cycle time, and 340 nm for discrete wavelength), but a similar instrument can be used instead.
3. The k_{cat} is calculated using the following formula:

$$k_{cat} = \frac{\Delta A \times 3.82}{6220 \times [\text{Protein}]}.$$

3.13 Direct UV Cross-Linking of Bound Nucleotides to Estimate ATP Binding

Short wavelength UV light (UV-A) damages nucleic acids and promotes formation of radicals that readily cross-link to amine groups. UV light can therefore be used as a near zero length covalent cross-linker of radiolabelled nucleotides to protein and has been used to demonstrate ATP binding to a number of ATP binding proteins (e.g., [23]). However, the resulting cross-link is not site-specific and nonspecific cross-linking can occur between the nucleotide and any accessible surface of a protein. Despite this drawback, this method does not require photo-cross-linking reagents (*see* Subheading 3.14) and the higher propensity of ATP to be bound within a specific ATP-binding pocket of an ATPase allows comparing relative binding affinities between related proteins and their mutant variants. Following the cross-linking reaction, the protein is separated from free nucleotides by SDS-PAGE and the radioactivity associated with the protein band of interest is quantified using Phosphor-Imaging. For PspF, the deletion of a conserved Walker B residue resulted in much diminished UV cross-linking compared with the wild-type protein (Fig. 3).

UV induced cross-linking is a rare event and requires high activity of the radiolabeled [α-^{32}P]ATP or [γ-^{32}P]ATP (e.g.,

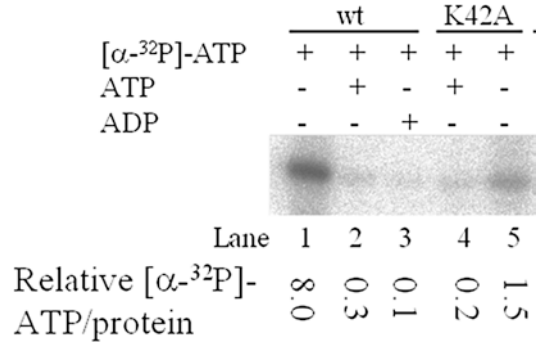


Fig. 3 UV cross-linking of [α-32P]ATP to $PspF_{1-275}$ WT and Walker motif A mutant K42A, in the presence and absence of competitor non-labelled nucleotides (as indicated). Relative [α-32P]ATP/protein levels were derived from phosphor-imaging compared to protein amounts (arbitrary units). Reproduced from Schumacher et al., 2004 [19] with permission from Elsevier Ltd

40 μCi/reaction) in order to detect and quantify the labelled protein species following SDS-PAGE. Note that when using [α-^{32}P]ATP, the resultant radiolabelled cross-linked species may well comprise both [α-^{32}P]ATP and [α-^{32}P]ADP due to hydrolysis during the cross-linking reaction. If the available protein concentration is limiting, samples may be concentrated by precipitation with 10 % (w/v) trichloroacetic acid after the cross-linking reaction and prior to electrophoresis. In order to avoid ATP hydrolysis during the reaction and possible heating of the sample exposed to strong UV-A irradiation, all steps should be carried out at 4 °C. The addition of an excess of non-radiolabelled ATP (mM range) can serve to establish the specificity of the cross-linking occurring at the ATP-binding site (Fig. 3 and *see* **Note 11**).

1. In a 20 μl of cross-linking buffer, mix protein and [γ-^{32}P]ATP to obtain a final concentration of 20 μM protein and 40 μCi [γ-^{32}P]ATP (e.g., 4 μl of 10 μCi/μl, 3,000 Ci/mmol). The negative control contains in addition the high concentration (3 mM final) of cold ATP to outcompete the radioactive one.
2. Transfer the reactions on a cooled flat surface as droplets. For instance, a Parafilm-covered glass plate can be placed on ice and the droplets pipetted onto the Parafilm.
3. Fix a UV-A light source 20 cm above the droplets and irradiate droplets evenly for 15–20 min (to increase reproducibility, switch on the UV light source at least 10 min before use).
4. Remove UV light source, and remove 16 μl of the droplets into SDS Laemmli buffer.
5. Heat for 3 min at 97 °C to ensure denaturation of protein.
6. Run SDS-PAGE of suitable polyacrylamide percentage (e.g., 12 %), using appropriate molecular weight markers but with caution due to the relatively high radioactivity of all solutions and components associated with the electrophoresis (*see* **Note 12**).
7. After fixing the SDS-PAGE gel (e.g., in methanol/acetic acid), wash the gel four times for several hours in a large volume of water or fixing solution (e.g., 1 l), so that all non-cross-linked nucleotides are quantitatively removed from the gel. This washing step will greatly reduce the radioactive background.
8. Stain (e.g., with Coomassie blue) and destain the SDS-PAGE gel as usual.
9. Vacuum-dry the SDS-PAGE gel.
10. Expose the dried gel to IP screen for 4–8 h.

3.14 UV Cross-Linking Using APAB

The *p*-azidophenacyl bromide (APAB, e.g., from Sigma Aldrich) is a photo-cross-linker that has a cross-linking radius of 9.7 Å. It can be strategically conjugated to phosphorothiolated DNA at designated sites [24].

1. Typically, DNA binding reactions are allowed to occur at 37 °C for 15 min in black Eppendorf tubes (for instance, using unlabelled core RNAP and σ^{54}, or the nucleotide analogue trapped complex).
2. Reaction mixtures are then UV-irradiated (with the Eppendorf lid open) at 365 nm for 1 min using for example a CL-1000 Ultraviolet cross-linker (UVP).
3. A 2 μl sample of the cross-linking mixture is directly loaded on a 4.5 % native gel (*see* Subheading 2.6).
4. The remaining reaction mixture is diluted with 4 μl of denaturing Cross-linking Dye, heated at 95 °C for 5 min and loaded on a 7.5 % SDS gel.
5. Both gels are dried and exposed to an IP plate for 1 h.

3.15 UV Cross-Linking Using pBpa

The *p*-benzoyl-L-phenylalanine (*p*Bpa, Bachem) is a photo-reactive artificial amino acid that can be genetically incorporated at any desired location (tagged by an amber stop codon) using site-directed mutagenesis and an orthogonal *Methanococcus jannaschii* tRNA-tRNA synthetase pair [25]. The *p*Bpa can cross-link to any C–H bond within 3.1 Å.

1. Typically, 0.3 μM *p*Bpa-tagged core RNAP is mixed with 2.35 μM un-tagged σ^{54} (or vice versa) at 37 °C for 15 min to allow stable complex formation.
2. Reaction mixtures are UV-irradiated in a CL-1000 Ultraviolet cross-linker at 365 nm on ice for 30–60 min and then divided for analyses by native gel (2 μl sample on a 4.5 % gel; *see* Subheading 2.6) and denaturing SDS gel (7 μl sample on a 7.5 % gel; *see* Subheading 2.9).
3. The dried gels are exposed to IP plates for 1 h and visualized.

3.16 Determining bEBP Oligomeric State by Gel Filtration

1. The oligomeric state of a bEBP can be established by determining the volume it elutes at in comparison to globular protein standards, which typically consist of five proteins: thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (66 kDa), and carbonic anhydrase (29 kDa). Two microliter of each protein (20 μM for the low concentration state and 50 μM for the high concentration state) are added together on ice with 10 μl of gel filtration buffer.

2. The 20 μl mixture is centrifuged at 15,000 rpm or 16,000 × *g* for 3 min to remove any particulates and transferred to a HPLC vial containing a 200 μl glass insert.
3. The vial is placed in the autosampler of the HPLC system (e.g., as a Thermo Scientific Surveyor plus) and is programmed to inject 15 μl of the sample into the gel filtration column (we use Phenomenex Yarra SEC-S 3000) set up with a guard column (we use a Phenomenex Security Guard). Flow rates will vary depending upon the column's pressure limit but when using the Yarra column, a flow rate of 1 ml/min is used.
4. The UV detector will produce a trace of the five standards which will act as a reference "ladder" for subsequent runs.
5. Samples are prepared in the same manner as the ladder. The buffer running through the column is the Gel filtration buffer which can include 0.4 mM of ATP or ADP to assess $PspF_{1–275}$'s ability to oligomerize in nucleotide conditions.

3.17 Manipulation of bEBP and Related Target Genes

Datsenko and Wanner [26] developed a simple method to delete target genes on the bacterial chromosome.

1. A FRT-*kan*-FRT cassette conferring kanamycin resistance from template plasmid pKD4 is amplified using hybrid primers containing the flanking sequences of the target gene and sequences homologous to 5′ or 3′ of the FRT-*kan*-FRT cassette.
2. The PCR product is electroporated into a strain carrying a temperature-sensitive helper plasmid encoding λ Red recombinase and transformants are grown on LB (*see* Subheading 2.2) agar (17 g/l) + kanamycin (25 μg/ml) plates for 24 h at 42 °C to lose the helper plasmid.
3. The gene deletion can be moved to strains of interest via phage $P1_{vir}$ transduction [27].
4. To generate a markerless gene deletion, the kanamycin cassette can be removed by transformation with the temperature sensitive plasmid pCP20 (*see* **Note 13**) encoding FLP enabling recombination at the FRT sites [28]. To eliminate pCP20 carrying kanamycin cassette after transformation and recombination at 30 °C overnight, the ampicillin resistant cells are grown on plane LB + agar plate overnight at 42 °C and then on LB + agar, LB + agar + kanamycin and LB + agar + ampicillin plates overnight at 37 °C. Cells sensitive to kanamycin and ampicillin are chosen (*see* **Note 14**).

   The λ Red recombineering method in combination with antibiotic/sucrose counterselection using the *nptI-sacB* system [29] can be used to replace a gene with a variant form at its native locus (*see* **Note 15**).

5. First, the gene of interest is replaced with the *nptI-sacB-kan* cassette conferring kanamycin resistance and sucrose (5 %) sensitivity then the *nptI-sacB-kan* cassette is replaced with the desired variant of the target gene through selection for resistance to 5 % sucrose and sensitivity to kanamycin.
6. The *nptl-sacB kan* cassette is PCR amplified with primers containing 5′- and 3′-flanking DNA sequences of the target gene and electroporated into a strain carrying a helper plasmid encoding the λ Red recombinase.
7. Transformants with the target gene replaced by *nptl-sacB-kan* are selected on kanamycin (25 μg/ml) plates at 42 °C to eliminate the temperature sensitive helper plasmid, then grown in liquid culture without selection, plated onto glucose and then re-streaked on sucrose (5 %) plates to select for sucrose-sensitivity.
8. The kanamycin resistant/sucrose sensitive strains are then re-transformed with the helper plasmid and electroporated with a PCR product encoding the variant of the target gene to replace *nptl-sacB-kan* through a second recombination event. Successful gene replacement is detected via a sucrose resistant/kanamycin sensitive phenotype.

3.18 Transcriptional Reporter

The bEBP-dependent promoter of interest including the upstream regulatory regions is placed in front of a reporter gene whose production can be monitored and correlated to promoter activity. One of the most commonly used reporter genes is *lacZ* encoding β-galactosidase. Hydrolysis of the colorless lactose analogue ONPG (*o*-nitrophenyl-*b*-D-galactoside) by LacZ results in production of the yellow orthonitrophenol (2,4-di-nitrophenol) [27]. The amount of orthonitrophenol produced is proportional to the number of LacZ molecules in the solution. Promoter activity can be quantified by measuring the interval between addition of ONPG and the color-change and is expressed as Miller units (MU).

1. After determining cell-density 50–500 μl of the bacterial culture is added to 500 μl Z buffer.
2. Following addition of 50 μl chloroform and vortexing for 30 s (cell lysis), the solution is incubated at 30 °C with 200 μl ONPG (4 mg/ml dissolved in Z buffer and pre-warmed to 30 °C).
3. Once the solution has turned yellow the reaction is quenched by 500 μl 1 M Na_2CO_3 and the time between addition of ONPG and quenching is recorded.
4. Cell debris and chloroform are pelleted by centrifugation and the absorbance of the supernatant at OD_{420} is measured.

5. Promoter activity is calculated based on the following equation:

$$\text{Miller Units}(\text{MU}) = (A_{420} \times 1{,}000) / (A_{600} \times t \times V).$$

A_{420} = absorbance of reaction at 420 nm; A_{600} = absorbance of culture at 600 nm; t = reaction time (minutes); V = volume of cells used in the assay (ml).

Use of LacZ as reporter also allows for a facile qualitative screen of factors impacting promoter activity when growing the cells on plates containing the lactose analogue X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or on the less sensitive McConkey agar (e.g., from Fisher Scientific; New Jersey). Colonies with high promoter activity will appear blue (X-gal) or red (McConkey). Native *lacZ* in the strains assayed may interfere with *lacZ*-based promoter activity studies. Various strains are available through the *E. coli* Genetic Stock Center at Yale University, USA in which expression of native *lacZ* is inhibited either through insertion of transcriptional terminators (e.g., BW25113) [26] or gene deletion (e.g., BW14894) [30].

3.18.1 Chromosomal Reporters

In vivo transcription is often studied using plasmid-borne reporters. Potential associated dose effects due to increased copy-number can be overcome by using strains lacking the *pcnB* gene [31, 32] or by integrating the reporter into the chromosome.

1. The promoter of interest is thereby fused to *lacZ* encoded on plasmid pRS415 [33].
2. Phage λBDC531 (*imm*^{21}) is grown on cells containing pRS415 with the reporter fusion.
3. The phage lysate is then used to generate lysogens in strain SA1943 identified on McConkey plates containing galactose and ampicillin (40 μg/ml) through the *gal*$^{-}$ phenotype and ampicillin resistance.
4. P1$_{vir}$ phage is grown on the SA1943 containing the λ(promoter-lacZ) lysogen and those lysates are used to transduce the recipient strain DY226.
5. The transductants are selected for ampicillin resistance at 42 °C and screened for a *bio*$^{+}$ phenotype and the presence of prophage (sensitivity to λ phages).
6. Finally, the promoter-*lacZ* fusion is moved to the desired strain via phage P1$_{vir}$ transduction selected for through ampicillin resistance.

While LacZ is typically used for end-point measurements, fluorescent proteins such as GFP as reporters allow for noninvasive time-course analyses of promoter activity along the growth curve

of bacterial cells. Simultaneous recording of cell densities and fluorescence emission from the reporter protein in a multi-well format facilitates a large scale high-throughput screen of promoter activity across a broad spectrum of conditions with minimal hands-on time. Plasmid-borne fluorescent transcriptional reporter are readily available, e.g., through the Standard European Vector Architecture (SEVA) [34]. Combination of fluorescent reporter with differing emission spectra further enables the activity measurement of more than one promoter at a time.

3.19 In Vivo Footprinting

In vivo footprinting can be used to study the interaction of the RNA polymerase-σ^{54} holoenzyme and bEBPs with the promoter in the context of the living cell.

For dimethyl sulfate (DMS) footprinting

1. DMS is added to 50 ml bacterial culture [cells are grown in exponential phase in rich media (LB, *see* Subheading 2.2) to $OD_{600} = 0.6$ at 37 °C] at a final concentration of 0.1 % (*see* **Note 16**).
2. Cells are incubated for 2 min, collected by centrifugation and washed twice with 150 ml of cold saline phosphate buffer.
3. Purified plasmid DNA prepared from the culture is resuspended in 20 μl of 1 M piperidine, heated at 90 °C for 30 min to cleave the DNA at methylated guanine residues, recovered by precipitation with ethanol, and washed with 70 % (v/v) ethanol.
4. DNA is dissolved in 32 μl water and 0.1 pmol primer 5′ end-labelled with [γ-^{32}P]ATP (3,000 Ci/mmol) is added to 8 μl of DNA in a final volume of 10 μl of 10 mM $MgCl_2$, 10 mM Tris–HCl pH 8.0. After boiling for 2 min the mixture is cooled on ice.

For potassium permanganate ($KMnO_4$) footprinting

1. $KMnO_4$ is added to 50 ml bacterial culture (as above, but a minimal media is needed to prevent extensive quenching of KMnO4) at a final concentration of 10 mM (*see* **Note 16**).
2. Cells are incubated for 5 min, collected by centrifugation and washed twice with 150 ml of cold saline phosphate buffer.
3. Plasmid DNA harboring the promoter of interest isolated from a 50 ml culture is dissolved in 150 μl of water.
4. 0.2 pmol primer 5′ end-labelled with [γ-^{32}P]ATP (3,000 Ci/mmol) is added to 50 μl of plasmid and the DNA denatured by adding 5.7 μl 10 mM NaOH, heating at 80 °C for 2 min, and cooling on ice.
5. For hybridization, the solution is neutralized by addition of 6.3 μl TMD and incubated for 15 min at the T_M for each primer.

6. After ethanol precipitation and washing, samples are resuspended in 10 µl water.

For primer extension

1. 2 µl of a freshly prepared Primer Extension mix is added to the footprinting reaction (DMS or $KMnO_4$).
2. The reaction is incubated for 10 min at 50 °C (after DMS treatment) or for 20 min at 37 °C (after $KMnO_4$ treatment) and quenched by adding 4 µl of formamide/dye.
3. Extension products are analyzed on a 9 % (w/v) sequencing gel.

3.20 Bacterial Two-Hybrid (Bacterial Adenylate-Cyclase Two-Hybrid, BACTH) Analysis

BACTH analysis can be used to study bEBP protein–protein interactions in vivo. One system [35] exploits the physical separation of the T18 and T25 fragments of adenylate-cyclase (Cya) from *Bordetella pertussis*. T18 and T25 can only form an active enzyme when in close proximity to each other. Effective reconstitution of Cya is a direct consequence of the interaction of two proteins fused to T18 and T25 and is reported via LacZ expression.

1. *E. coli* BTH101 (*cya*$^-$) (*see* **Note 17**) is co-transformed with the two plasmids encoding the proteins of interest fused to the N- or C-terminus of T18 and T25 respectively.
2. Cells are grown in LB (*see* Subheading 2.2) at 30 °C and expression of the fusions is induced with 0.5 mM IPTG.
3. After 1 h, LacZ activity is measured by β-galactosidase assays [27]. A ≥2-fold increase in LacZ activity compared to the negative control is scored as positive interaction. Cells containing pUT18C-zip and pKT25-zip (zip: GCN4 leucine-zipper) serve as positive control [35].

BACTH experiments with Hrp proteins (performed at 25 °C)

1. The transformants are plated onto LB + agar + ampicillin (100 µg/ml) + kanamycin (50 µg/ml) + X-Gal (40 µl/plate of 20 mg/ml stock) + IPTG (0.5 mM) medium and incubated at RT for 2 days.
2. Bacteria are grown in LB broth supplemented with ampicillin (100 µg/ml) and kanamycin (50 µg/ml) at 25 °C overnight, cultures are then diluted 100-times, let to grow until $OD_{600} = 0.4$, and induced with 0.5 mM IPTG for next 3 h (*see* **Note 18**).
3. LacZ activity is measured by β-galactosidase assays as above.

HrpR-HrpS Three Component BACTH

To characterize interaction properties among three Hrp proteins we performed BACTH three component system [3, 36]. In addition to the T18C-hrp and T25-hrp fusions a third component is

inserted between the *Kpn*I-*Eco*RI sites of pUT18C-hrp vector downstream of the T18C-hrp fusion) and co-expressed as wild type protein. Transformations and β-galactosidase assays are performed as described above.

3.21 Analysis of bEBP Protein Dynamics In Vivo

Engagement in interactions with DNA, e.g., the binding of bEBPs to UAS of σ^{54} promoters, may affect the dynamics of proteins [8]. The effects on protein dynamics can be studied through live cell imaging via epifluorescence microscopy.

1. Cells are grown in a minimal medium at 30 °C to $OD_{600} = 0.8$.
2. Foci of the fluorescently labelled protein of interest are localized with sub-pixel accuracy, joined to tracks comprising 200–1,000 foci and the mean square displacement (MSD) for different time lags is calculated using for example Matlab (Mathwork) scripts based on the algorithm by Crocker and Grier [37].
3. Protein dynamics are expressed as diffusion coefficients which are calculated as the $1/4^{th}$ of the slope of the MSD curve.

3.22 Analysis of Protein Stoichiometries In Vivo

We developed a general method for measuring stoichiometries of protein complexes in vivo [38].

1. Cells are grown in N^-C^- minimal medium at 30 °C to $OD_{600} = 0.8$.
2. Bacterial cells expressing the fluorescently labelled protein of interest are imaged through wide-field fluorescence microscopy. Fluorescent foci are photobleached to single-molecule intensity by continuous excitation of the sample, e.g., through an excitation beam of 3 mW (before entering the microscope). The photobleaching sequence can be recorded in thousand 500×500 pixel frames at a rate of 25 frames/s. The signal-to-noise ratio of the photobleaching intensity trace is improved by applying an edge-preserving nonlinear digital filter based on the Chung–Kennedy algorithm [39] to effectively discard additive Gaussian-like noise.
3. The signal is then used to calculate the Pairwise Difference Distribution Function [40, 41] and its power spectrum.
4. The peaks in the power spectrum are detected and the number of steps present in the original photobleaching trace is determined.
5. The stoichiometry of the fluorescently labelled protein is then determined through the number of fluorophores present in the foci calculated as the quotient of the background- subtracted initial fluorescence intensity and the photobleaching step.

4 Notes

Troubleshooting in vitro assays typically involves ensuring nucleotides for RNA synthesis are not degraded, protein preparations and buffers are RNAse free and that DNA templates are intact. Careful attention to storing reagents at −20 °C, not freezing and thawing reagents more than one or two times is a key.

1. The presence of $MgCl_2$ in protein sample and Buffers A/B-2 is crucial for core RNAP and σ^{54} to bind to the heparin column.
2. Kinetic considerations: The ATPase activity of bEBPs depends on the oligomeric—probably hexameric—state of the proteins and the nucleotide occupancy within the oligomer [42]. For instance it was shown for the hexameric PspF AAA+ domain that low ADP concentrations (0.1 mM) increases the overall V_{max} of the complex and that ATP concentrations above 2 mM can repress its overall V_{max} [11]. Further, it was shown that under defined conditions V_{max} is reached between a 1–1 and a 2–1 ratio between ADP and ATP [20]. These effects are caused by nucleotide state dependent allosteric effects on ATP hydrolysis between subunits of the hexameric assembly, a feature that probably operates more generally between bEBPs subunits. To determine V_{max}, it is therefore important to determine hydrolysis rates over a range of protein concentrations as well as ATP concentrations. First, a protein titration experiment should be carried out at a constant saturating ATP concentration in the millimolar range (e.g., 1 mM, the dissociation constants of PspF for ATPγS and ADP are 34 and 118 μM, respectively [11]).The protein range over which the turnover rate (k_{cat}) is constant would suggest that ATPase competent oligomers are formed. Then, using a constant protein concentration at the higher end of this range, an ATP titration experiment can be used to determine V_{max}. It is also advised—considering the potential stimulatory effect of ADP to the k_{cat} of PspF and other bEBPs—to maintain conditions in which only a small proportion of the ATP has been hydrolyzed to ADP (e.g., <20 % of total input ATP) when determining initial hydrolysis rates.
3. The [α-^{32}P]ATP (10 μCi/μl, 3,000 Ci/mmol) contribution to the final ATP concentration is negligible as the [α-^{32}P]ATP concentration in the ATP mix is 0.1 μM (e.g., 0.6 μCi/μl and 3,000 Ci/mmol).
4. The bEBP must be of high purity, not to contain ATP, ADP (shown by UV absorbance at 254 nm).
5. The cellulose based absorbant may be scratched off along the vertical lines, for instance with the edge of a fine spatula. This stops otherwise possible migration between lanes and increases

the number of lanes that can be loaded on one TLC sheet (up to 20 lanes of 1 cm width).

6. Each protein sample should migrate in a straight channel (width–height: 1 cm:20 cm). To create the boundaries of each migration channel, one should use a sharp object to cut through the TLC powdery surface to reach the plastic sheet at the bottom.
7. Placing the sheet with the absorbant upwards near the draft of a fume cupboard accelerates drying.
8. Crystallization of the 0.4 M K_2PO_4/0.7 M boric acid solvent can significantly retard sample migration. Should this occur, make the fresh solvent.
9. Exposure duration should be chosen to ensure detection within the linear range of the Phosphor-imaging setup.
10. Assuming protein stability, taking several suitable time points from each sample allows obtaining data points for initial turnover rates, from which k_{cat} can be derived by dividing by the time of reaction.
11. While this control is imperfect, it can be assumed that non labelled ATP would readily out-compete binding of radiolabelled ATP to the ATP binding site but not at nonspecific sites.
12. Due to the high radioactivity requirements in these assays, the SDS PAGE gel and the electrophoresis buffers will contain high radioactivity levels (be very "hot"). It is advisable to initially run the SDS-PAGE until the blue Laemmli dye has migrated to half of the length of the gel and to then exchange the electrophoresis buffer, disposing the initial, now radioactive buffer, adequately.
13. Plasmid pCP20 (Ampicillin resistance) should always be kept and/or propagated for its isolation in a strain lacking any kanamycin or chloramphenicol cassette.
14. The ampicillin and or kanamycin sensitivities and resistances should be always checked using LB medium + antibiotics growth tests with positive and negative controls.
15. Strains lacking chromosomal *rpoN* are available through the *E. coli* Genetic Stock Center at Yale University, USA (*rpoN208*::Tn10; *rpoN730*(del)::kan) enabling in vivo analyses of Sigma 54 variants in the absence of its native form.
16. To trap the σ^{54}-dependent promoter in its open complex form, rifampicin is added to the growing culture at a concentration of 200 μg/ml 10 min prior to the footprinting reaction.
17. Before every experiment a BTH101 (cya-) strain should be checked for its LacZ-phenotype (white colonies) on LB + agar + X-gal plate. A white colony will be chosen for the

overnight culture in LB medium for preparation of the competent cells.

18. The appropriate transformants are grown in triplicate LB medium in the presence of 0.5 mM IPTG and 100 μg/ml ampicillin and 50 μg/ml kanamycin at 25 °C for 20 h and extents of interactions between different hybrid proteins are then quantified by measurement of β-galactosidase activity.

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